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GC-MS/MS ANALYSES OF BIOLOGICAL SAMPLES IN SUPPORT OF DEVELOPMENTAL TOXIC EFFECTS ON WHOLE-BODY EXPOSURE OF RATS TO GB

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This report documents the results of gas chromatography tandem mass spectrometry analyses of blood, tissues, and organs (heart, lung, liver, kidney, brain, eye, and diaphragm), which were obtained from rats (postnatal days 14, 21, 42, and 70) after their exposure to various concentrations of sarin (GB) using the whole-body inhalation system for 60 min. The amount of free and regenerated GB present in these biological samples was quantified using chemical ionization mass spectrometry with isotope dilution.

15. SUBJECT TERMS

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PREFACE

The work described in this report was authorized under project no. BARDA CBRN OS 75442 ASPR-11-04601 and Institutional Animal Care and Use Committee (IACUC) Protocol 12-447. The work was started in October 2012 and completed in December 2014 as recorded in U.S. Army Edgewood Chemical Biological Center (ECBC) Notebooks 09-0088, 13-0001, and 13-0080.

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This report has been approved for public release.

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CONTENTS

FIGURES

TABLES

GC-MS/MS ANALYSES OF BIOLOGICAL SAMPLES IN SUPPORT OF DEVELOPMENTAL TOXIC EFFECTS ON WHOLE-BODY EXPOSURE OF RATS TO GB

1. INTRODUCTION

Chemical warfare nerve agents (CWNAs) include G-agents such as tabun (ethyl *N*,*N*-dimethylphosphoramidocyanidate; GA), sarin (isopropyl methylphosphonofluoridate; GB), soman (pinacolyl methylphosphonofluoridate; GD), and cylcosarin (cyclohexyl methylphosphonofluoridate; GF), as well as less-volatile V-agents such as VX (*O*-ethyl *S*-[2-diisopropylaminoethyl] methylphosphonothioate) and Russian VX (*O*-isobutyl *S*-[(2-diethylamino)ethyl] methylphosphonothioate). Like organophosphorus (OP) pesticides, CWNAs exert their toxicological effects by inhibiting acetylcholinesterase (AChE), the enzyme responsible for the degradation of the neurotransmitter acetylcholine (ACh) in the central and peripheral nervous systems. ACh accumulation within the synaptic cleft after extensive AChE inhibition increases and prolongs the stimulation of muscarinic and nicotinic receptors on autonomic ganglion, end-organs, myocytes and postsynaptic neurons, which leads to an acute cholinergic crisis that is characterized by autonomic and cardiac dysfunction, involuntary movements, miosis, muscle fasciculations, respiratory distress and seizures (Russell and Overstreet, 1987).

In the event of a mass casualty situation involving the release of CWNA amongst a civilian population or the military community, pregnant women, infants and small children are likely to be exposed. For example, four pregnant women between 9 and 36 weeks of gestation were admitted to the hospital with mild cholinergic symptoms after the release of GB in the Tokyo subway system (Ohbu et al., 1997). In fact, infants and small children may be at greater risk for inhalation and dermal exposures to CWNA than adults because of greater minute ventilation rates (Bloomfield, 2002) and larger surface area to body mass ratios (Guzelian et al., 1992), respectively. Additionally, infants and small children are considered to be at greater risk for seizures as compared with adults (Ben-Ari and Holmes, 2006), and early-life seizures increase a child's susceptibility to seizure-induced brain damage in adulthood (Thompson and Wasterlain, 1997).

The human perinatal period is of particular concern in neurotoxicological evaluations because developmental exposure to xenobiotics during this time may disrupt critical neuronal processes (e.g., apoptosis, differentiation, migration, myelination, proliferation, and synaptogenesis) and produce effects that differ dramatically from those produced by adult exposures (reviewed in Rice and Barone, 2000). Several well-documented examples of xenobiotics that produce developmental neurotoxicity in animals and humans at levels that do not cause neurotoxicity in adults include ethanol, lead, methylmercury, phenytoin, and polychlorinated biphenyls (Francis et al., 1990).

The developing nervous system is particularly vulnerable to xenobiotics that disrupt the cholinergic system, which plays a fundamental role in neuronal differentiation and proliferation, synaptic development and function, and ultimately behavioral performance

(Slotkin, 2004). Pope et al. (1991) demonstrated that neonatal rats (postnatal day [PND] 7) were more sensitive than adult rats (PNDs 80–100) to the neurotoxic effects of a variety of OP pesticides, including chlorpyrifos (CPF).

Compared with CPF, there have been relatively few studies on the developmental neurotoxicity of CWNA exposure. Sterri et al. (1985) showed that PND 5 rats were more sensitive than PND 30 rats to the neurotoxic effects of GD. Shih et al. (1990) showed that PND 30 rats had less weight loss and more-rapid growth recovery after GD exposure than that of older rats. Given that the youngest rat tested in this study was 30 days old, it is probable that if even younger rats were exposed at critical periods in neuronal development, they would have greater susceptibility to the neurotoxic effects associated with CWNA exposure. Furthermore, the time-to-onset and the severity of toxic signs associated with CWNA exposure depend, not only on the animal species, dose, and gender, but also on the route of exposure (Mioduszewski et al., 2002). In most laboratory experiments, CWNAs are administered via a subcutaneous injection. In a battlefield or terrorist situation, however, the most relevant route of exposure is whole-body (primarily inhalation) for GB.

Therefore, *Developmental Toxic Effects on Whole-Body Exposure to Chemical Warfare Nerve Agents (CWNA) in Rats: Effects on Brain and Behavior*, the Institutional Animal Care and Use Committee (IACUC) protocol number 12-447 (IACUC, 2012) provides additional data needed to bridge the gap between these routes of exposure, especially at younger ages, to make more accurate human risk assessments. In addition, physiologically based pharmacokinetic pharacodynamic (PBPK/PD) modeling, which was incorporated into the study, yields a quantitative basis for extrapolating animal-to-human exposure conditions and predicting the human response to a chemical of interest. This report documents the results of the gas chromatography–tandem mass spectrometry (GC-MS/MS) analyses that were used to quantify the amount of free and regenerated GB (r-GB) present in blood, tissues, and organs (heart, lung, liver, kidney, brain, eye, and diaphragm). These results provide the data needed for the PBPK/PD modeling.

2. METHODS

2.1 Animal Exposures

2.1.1 Chemical Materials

GB was obtained from U.S. Army Edgewood Chemical Biological Center (ECBC) chemical agent standard analytical reagent material (CASARM) stock (GB-U-5340-CTF-N). Before use, the CASARM-grade GB was verified with quantitative ^{31}P nuclear magnetic resonance (NMR) spectrometry as 98.91 ± 0.92 wt % (notebook 12-0047-84).

2.1.2 Inhalation Exposure System

Animal exposures to CWNAs were conducted in a 1000 L dynamic airflow inhalation chamber at ECBC. The Rochester-style chamber is constructed of stainless steel with

glass or Plexiglas windows on each of its six sides. The interior of the exposure chamber is maintained under negative pressure (0.2–0.3 in. of water) as monitored with a calibrated magnehelic differential pressure gauge (Dwyer Instruments; Michigan City, IN). For GB vapor generation, a Harvard Pump 11 Elite syringe pump (Harvard Apparatus; Holliston, MA) was used to deliver liquid agent into a spray atomizer, where it was mixed with compressed air to form vapor. Agent-exposure groups consisted of 10–20 rats placed in mesh cages during the 60 min exposure. Chamber airflow and temperature were monitored continuously during the exposure period, and relative humidity was measured at the beginning and at the end of the exposure.

Two sampling methods were used to monitor and analyze the nerve agent vapor concentration in the exposure chamber. The first method was quantitative and used solid sorbent tubes (Tenax/Hayesep; CAMSCO, Houston, TX) to trap nerve agent, followed by thermal desorption and GC analysis (Agilent 6890; Agilent Technologies; Wilmington, DE). Samples were drawn from the chamber every 10 min, with each draw lasting 2 min. The second method was a continuous-monitoring technique that used a real-time phosphorous monitor (HYFED, model PH262; Columbia Scientific Industries Corporation; Austin, TX). Output from the HYFED monitor provided a continuous strip chart record of the rise, equilibrium, and decay of the chamber vapor concentration during an exposure.

2.2 Sample Preparation and Analysis

2.2.1 Chemical Materials

GB (GB-U-5340-CTF-N) and $^{2}H_{6}$-GB were obtained from ECBC CASARM stock. Before use, the CASARM-grade GB was verified with quantitative ^{31}P NMR spectrometry as 93.8 ± 2.4 wt % (notebook 11-0003-77), and the $^{2}H_{6}$-GB was verified as 24.86 ± 0.37 wt % (notebook 11-0003-113). Potassium fluoride (KF), 2-propanol (IPA), ethyl acetate, glacial acetic acid, and anhydrous sodium sulfate were obtained from Sigma-Aldrich (St. Louis, MO) at ≥99% purity. Sodium acetate was purchased from Fischer Chemicals (Fair Lawn, NJ) at >99% purity. Ammonia and methane were obtained from Sigma-Aldrich, and helium was obtained from Messer (Malvern, PA) at purities >99.9%.

2.2.2 Stock Solutions and Calibration Standards

Stock solutions of GB and $^{2}H_{6}$-GB (internal standard [IS]) were prepared in IPA at concentrations of 2.188 mg/mL (notebook 11-0003-84-01) and 0.519 mg/mL (notebook 11-0003-109-01), respectively, and stored at –20 °C until used. Working solutions (5–10 µg/mL) were prepared by diluting the stock solutions in ethyl acetate. Calibration standards of GB were prepared by diluting the working solution to obtain the following 12 concentration points: 0.5, 1, 5, 10, 25, 50, 100, 200, 400, 600, 800, and 1000 ng/mL (notebooks 11-0003-116-04 through 11-0003-116-15). Each calibration standard also contained 200 ng/mL $^{2}H_{6}$-GB, diluted from the working solution. All calibration standards were stored at –20 °C until analysis. Figure 1 shows a typical calibration curve with "Relative Response" defined as $Area_{GB}/Area_{IS}$ and "Relative Concentration" defined as $Concentration\ (ng/mL)_{GB}/Concentration\ (ng/mL)_{IS}$. A quadratic curve

fit was used with a 1/× weighting factor to yield a correlation of $R^2 = 0.9994$ over 3 orders of magnitude.

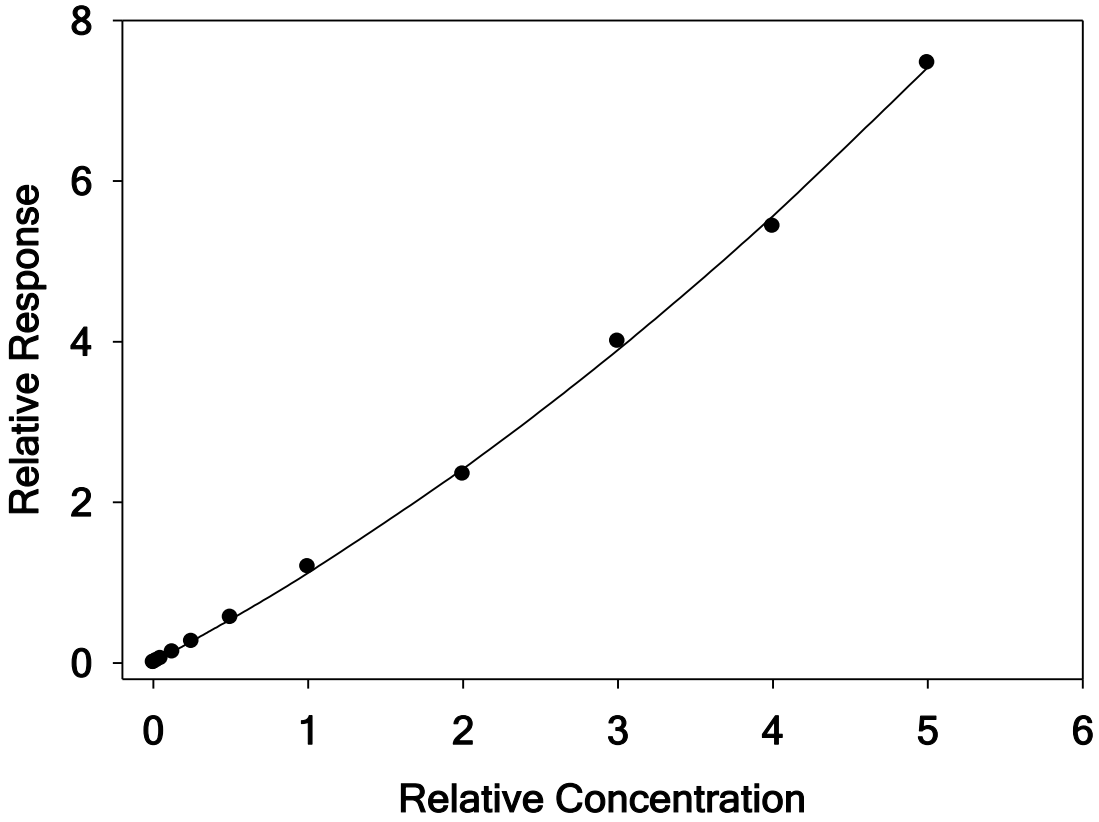

Figure 1. GB calibration curve using 12 calibration standards.

2.2.3 Analytical Method

Sample assays were performed using an Agilent Technologies 7000A GC/MS Triple Quad instrument (Wilmington, DE). Gas chromatographic separations were achieved using a Restek (Bellefonte, PA) RTx-1701 column (30 m × 0.25 mm i.d., 0.25 µm film thickness). The carrier gas was helium with a flow rate of 1 mL/min. Injections of 2.0 µL were made using an autoinjector (7693 ALS, Agilent Technologies) into a splitless injector port at a temperature of 250 °C. The initial oven temperature of 35 °C was held for 1 min, then ramped at 50 °C/min to 50 °C, ramped again at 2 °C/min to 64 °C, and finally ramped again at 50°C/min to 200 °C. The column was then backflushed at 280 °C for 4 min at reduced inlet pressure (–6.3 mL/min). Figure 2 shows a typical multiple reaction monitoring (MRM) mode chromatogram with retention times for GB (9.819 min) and $^2H_6$-GB (9.772 min) noted.

Samples were ionized using positive-ion chemical ionization (CI) with ammonia reagent gas. CI source conditions were optimized using Fluoroether E3 (Chemical Abstract Service [CAS] registry number: 3330-16-3, Agilent Technologies) tuning compound with

methane reagent gas. Mass spectra were obtained at a dwell time of 0.2 s for each transition in the MRM mode. Helium was used as the collision gas with collision energy (CE) of 10 V. The CE was optimized for the mass-to-charge ratio (*m/z*) 158 > 99 transition for GB and the *m/z* 164 > 100 transition for $^{2}H_{6}$-GB. The MassHunter software provided with the 7000A system was used to process and analyze the data. The software provides automated peak detection, calibration, and quantitation.

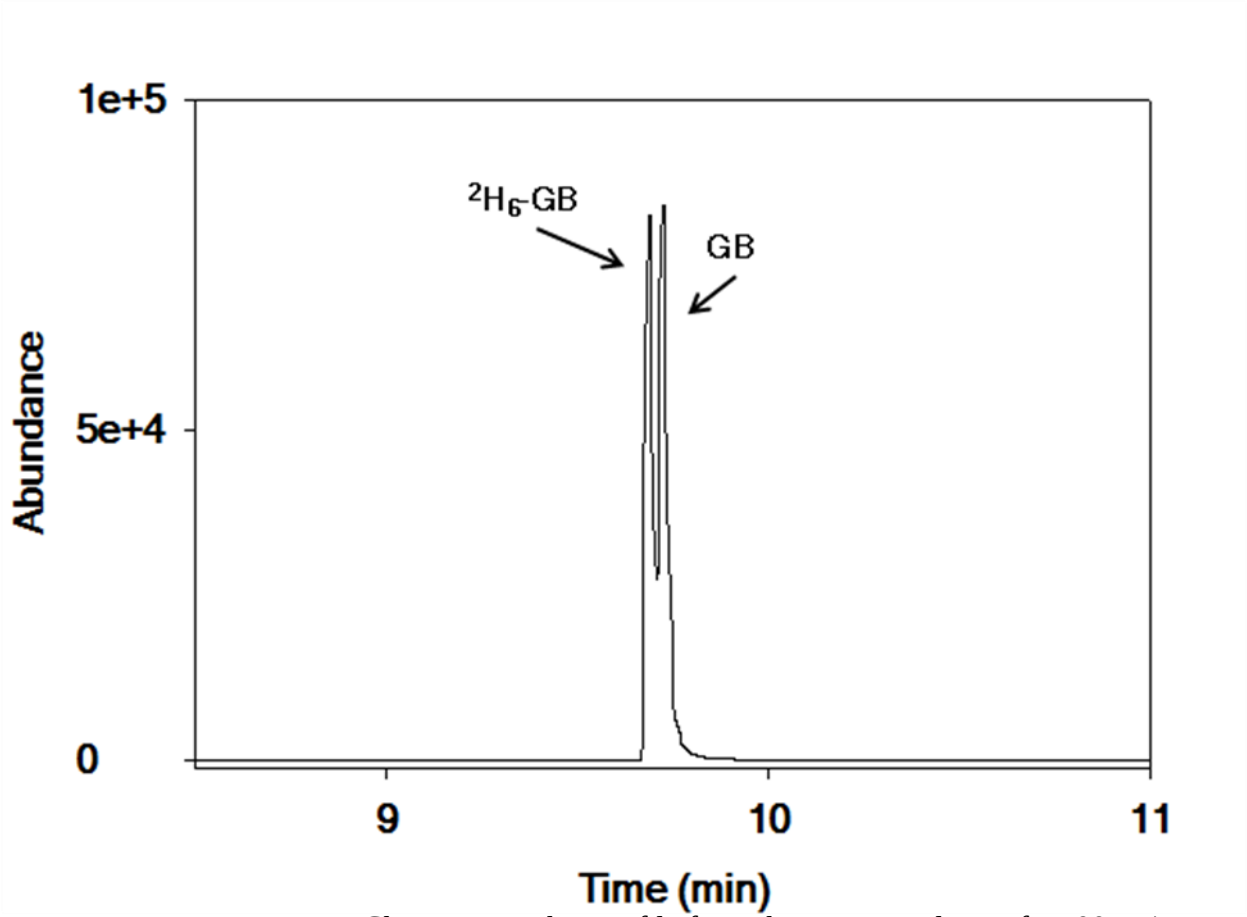

Figure 2. Chromatographic profile from the MRM analysis of a 200 ng/mL calibration standard.

2.2.4 Sample Preparation

Sample preparations for this study were similar to those published by McGuire et al. (2008). Upon arrival, all biological samples were stored at –80 °C until analyzed. Whole blood samples were extracted for GB using Sep-Pak Vac 3 cc (200 mg) C18 solid-phase extraction (SPE) cartridges (Waters Corporation; Milford, MA), which were first conditioned with 1 mL each of ethyl acetate, IPA, and then pH 3.5 acetate buffer (0.01M sodium acetate and 0.2M glacial acetic acid). After weighing a sample of blood in a 2.0 mL microcentrifuge tube (Sigma-Aldrich; St. Louis, MO), 1 mL of acetate buffer, 200 µL of KF solution (6M), and 1 µL of internal standard, $^{2}H_{6}$-GB were added. The mixture was vortex-mixed for 10–20 s and then centrifuged at 15,000 rpm for 5 min using a Micromax Microcentrifuge (Thermo IEC; Needham Heights, MA). After transferring the supernatant liquid to the SPE cartridge, the sediment at the

bottom of the microcentrifuge tube was resuspended with 750 µL of acetate buffer and 200 µL of KF solution. This mixture was also vortex-mixed and centrifuged and the resulting liquid was added to the original solution. After adding the mixture to the SPE cartridge, it was allowed to drain under a gentle vacuum. The analytes were eluted with 1 mL of ethyl acetate, which was collected and dried over anhydrous sodium sulfate. The ethyl acetate was withdrawn from the collection tube and filtered through a 0.2 µm nylon Acrodisc syringe filter (Pall Gelman Laboratory; Ann Arbor, MI) into a GC autosampler vial (Agilent Technologies), then concentrated to 50 µL for analysis.

Tissue and organ sample extracts were prepared in a similar manner employing freeze-fracture pulverization under cryogenic temperatures before the SPE extraction step. Using a Tissue Cryoprep system (Covaris, Woburn, MA), 0.5–1 g of tissue was pulverized. The pulverized sample was then mixed with 1 mL of acetate buffer, 200 µL of KF solution, and 1 µL of internal standard. It was then subjected to focused acoustics using an S-series focused acoustic energy system. This process causes precisely controlled cavitation and acoustic streaming at the focal point within the sample treatment vessel in a noncontact, isothermal process. After centrifugation at 4500 rpm for 15 min using a Sorvall Legend X1R centrifuge (Thermo Fisher Scientific Inc.; Waltham, MA), the supernatant liquid was transferred to the SPE cartridge, and the sediment at the bottom of the sample tube was resuspended with 750 µL of acetate buffer and 200 µL of KF solution. This mixture was vortex-mixed and centrifuged, and the resulting liquid was added to the original solution. Further sample processing was performed in a manner that was identical to that of the blood samples.

3. RESULTS AND DISCUSSION

The following results have been recorded in ECBC notebooks 09-0088, 13-0001, 13-0080, and 14-0084. Table 1 summarizes the data from the GB assays of whole blood and various tissues and organs obtained from PND 14 rats after a 60 min, whole-body inhalation exposure to GB. The chamber concentrations are indicated, along with the sampling times for the biological samples. For each type of biological sample, a control (only exposed to air) sample was spiked with GB at a concentration of 100 ng/g to determine the percentage of GB recovered. As indicated, these recoveries ranged from 93 to 127%. Tables 2, 3, and 4 show similar results from analysis of the 60 min, whole-body inhalation exposures to GB for additional age groups.

As expected from an inhalation exposure, the lung and eye tissues contained the majority of GB accumulated. A two-way analysis of variance (ANOVA) was done to test whether a significant difference was present between the male and female populations at the 1, 4, or 24 h sampling points for each of the PND rat groups. Using the means of regenerated and free GB for each biological sample from the same sampling time, testing results indicated that no statistically significant difference ($P < 0.05$) was present among the two genders at the three sampling times. The ANOVA testing was then extended to include the age of the rats in a three-way analysis. Again, no statistically significant difference was present. Because the GB exposure concentration for each age group was adjusted to produce a 0.6 LCt_{50} (lethal concentration with 50% chance of causing death) for a 60 min exposure, these results are not surprising when factors such as size and breathing rates are taken into account. This study also afforded a limited opportunity to examine the distribution and elimination of GB in rodents. Within the first hour,

GB was distributed to the brain (indicative of crossing the blood–brain barrier), heart, diaphragm, kidneys, and liver. An examination of the free and regenerated GB (r-GB) concentration in the liver samples obtained from the PND 21 rats indicated a first-order decline with half-lives of 1.7 h for the PND 21 males and 1.4 h for the PND 21 females. A similar analysis of the decline in the r-GB concentration found in the kidneys indicated half-lives of 15–20 h.

Because the goal of this report was to document the results of the GC-MS/MS analyses, no further interpretations were attempted. All data have been transferred to Dr. Jeffery M. Gearhart of the Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc. at Wright-Patterson Air Force Base for further efforts to develop the appropriate PBPK/PD models to predict the biological impact of GB exposure in young animals.

Table 1. Results from Regenerated and Free GB Assays of PND 14 Rats

Rat No.	Sex	Sample Time (h)	Concentration (mg/m^3)	Regenerated and Free GB (ng/g)							
				Whole Blood	Heart	Lung	Liver	Kidney	Brain	Eye	Diaphragm
436	F	24	1.34	3.7709	0.9973	43.3981	2.0923	0.2604	0.9427	10.9481	0.3275
437	F	24	0	93%*	BDL	BDL	BDL	BDL	BDL	BDL	BDL
438	F	1	1.78	4.2344	1.8749	63.3460	4.2231	0.7183	3.1216	17.8925	BDL
439	F	1	1.34	3.5614	1.5177	50.5535	0.7020	0.3570	1.9517	10.6679	BDL
440	F	24	1.78	3.4173	1.6964	62.6593	10.5085	0.3849	1.2706	10.5014	BDL
441	M	24	1.34	3.0325	1.1519	26.5174	3.0497	0.2319	1.2416	12.8186	BDL
442	M	1	1.78	NS	2.2936	44.2848	4.3589	0.9751	1.3415	17.8464	BDL
443	M	1	1.34	NS	1.3627	65.1554	6.1714	0.5505	1.6723	13.2349	0.3764
444	M	24	1.78	3.2008	1.8164	40.9100	7.1652	0.6776	1.5357	13.4423	0.2814
445	M	24	0	BDL	111%*	BDL	BDL	127%*	127%*	BDL	BDL
446	F	24	1.34	3.2598	0.3996	38.7501	0.3758	0.2365	0.5225	BDL	BDL
447	F	24	0	BDL	BDL	114%*	BDL	BDL	BDL	98%*	BDL
448	F	1	1.34	NS	0.1836	45.9704	2.5640	0.9227	3.2922	20.1592	0.7354
449	F	1	1.78	NS	3.2993	60.5673	5.7867	1.0825	4.7506	33.3005	BDL
450	F	24	1.78	5.7018	1.8194	50.0509	2.9573	0.6357	1.9018	13.8790	2.1095
451	M	1	1.34	NS	1.0960	44.6035	0.9163	0.3061	1.2086	BDL	0.3423
452	M	24	0	BDL	BDL	BDL	127%*	BDL	BDL	BDL	96%*
453	M	24	1.34	3.3817	0.6228	37.0311	0.4429	0.2078	0.4510	BDL	BDL

*Selected control samples (0 mg/m^3) were spiked with GB to determine percentage recovered.
BDL: Below detection limit (<0.05 ng/g)
NS: No sample was received.

Table 2. Results from Regenerated and Free GB Assays of PND 21 Rats

Rat No.	Sex	Sample Time (h)	Concentration (mg/m^3)	Regenerated and Free GB (ng/g)							
				Whole Blood	Heart	Lung	Liver	Kidney	Brain	Eye	Diaphragm
507	F	24	2.33	4.5781	0.7395	63.9194	4.7305	0.3825	0.8766	19.8321	0.2063
509	F	4	2.33	10.4983	0.7379	64.1355	3.5912	0.5535	0.6760	14.5953	BDL
510	F	1	2.33	12.1038	2.2787	159.5752	27.7532	0.7284	4.3375	25.1424	BDL
511	M	24	0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
512	M	1	2.33	9.6543	0.2951	22.5806	0.5928	0.0838	0.0841	12.1359	BDL
515	M	4	2.33	8.0851	0.6500	32.0158	3.2447	0.1363	0.1943	15.8853	BDL
516	F	24	2.33	4.4207	0.1757	30.7406	3.9724	0.1945	0.1496	8.9686	BDL
519	F	24	0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
522	M	4	2.33	5.9563	0.5496	53.4057	2.0892	0.2326	0.2718	11.7328	BDL
524	M	24	2.33	3.6268	0.1192	33.1844	0.6656	0.1648	0.0857	12.7324	BDL
525	M	1	2.33	7.8721	1.4239	88.1215	18.0470	0.5512	2.0174	18.9796	BDL
526	F	24	0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
528	F	1	2.33	6.8573	0.4464	50.8729	0.9039	0.2620	0.5235	7.6922	BDL
532	M	24	2.33	2.3147	BDL	11.7989	0.2439	0.0628	BDL	4.0993	BDL
534	M	24	0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
537	F	1	2.33	5.4314	0.7607	46.2432	2.0013	0.1611	0.4536	8.0905	BDL
539	F	4	2.33	5.9937	0.4535	53.5403	1.7869	0.2527	0.3809	19.6576	BDL
541	M	24	0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
544	M	4	2.33	4.3420	0.2116	18.5941	0.3623	0.0739	BDL	11.4787	BDL
546	F	24	2.33	2.2989	BDL	19.9333	0.1943	BDL	BDL	5.7905	BDL
547	F	24	0	NS	BDL	BDL	BDL	BDL	BDL	BDL	BDL
549	F	4	2.33	7.8153	0.6108	52.3146	1.4163	0.2822	0.4589	10.4172	BDL
553	M	1	2.33	3.9296	0.2366	13.7429	0.2062	BDL	BDL	11.0172	0.0956
554	M	24	2.33	2.6887	BDL	12.9346	0.2308	0.0933	BDL	8.5692	BDL

BDL: Below detection limit (<0.05 ng/g)
NS: No sample was received.

Table 3. Results from Regenerated and Free GB Assays of PND 42 Rats

Rat No.	Sex	Sample Time (h)	Concentration (mg/m^3)	Regenerated and Free GB (ng/g)							
				Whole Blood	Heart	Lung	Liver	Kidney	Brain	Eye	Diaphragm
456	F	1	2.25	3.4537	0.6410	28.8955	0.5290	0.4835	0.0995	13.2113	0.9659
459	F	24	0	BDL	131%*	BDL	BDL	BDL	111%*	BDL	BDL
460	F	4	2.25	4.1630	1.6715	58.8531	3.1648	0.7669	1.0148	18.9165	0.5053
461	M	24	2.25	2.3434	0.1979	0.1778	1.5113	32.4607	0.0701	9.9159	BDL
463	M	1	2.25	2.9763	0.4212	27.5962	0.4252	0.5243	0.0667	12.0388	0.0763
465	M	24	0	BDL	BDL	176%*	BDL	BDL	BDL	BDL	BDL
466	F	24	2.25	2.2841	0.2681	15.5452	0.5949	0.1326	BDL	3.2701	BDL
469	F	24	0	BDL	BDL	BDL	131%*	BDL	BDL	BDL	BDL
471	M	4	2.25	6.8971	0.4871	56.6182	6.8634	1.3098	0.2961	8.2449	BDL
475	M	1	2.25	NS	0.2823	18.4076	0.3925	0.3668	BDL	7.0733	BDL
478	F	1	2.25	8.2074	0.9648	64.4762	2.8064	1.0672	1.0739	16.4049	0.2687
480	F	24	2.25	2.3375	0.1785	10.6600	0.5945	0.1391	BDL	4.8045	BDL
482	M	24	0	BDL	BDL	BDL	BDL	127%*	BDL	BDL	BDL
484	M	24	2.25	2.7075	0.1779	13.9509	0.3543	0.1134	BDL	4.4711	BDL
488	F	4	2.25	5.9695	1.4713	92.8354	2.4689	0.8503	0.9369	11.6918	0.0568
489	F	24	0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	98%*
490	F	1	2.25	5.8970	0.2613	24.7277	0.4687	0.3685	0.0989	6.7872	BDL
492	M	4	2.25	11.0026	1.4666	156.4675	11.9909	1.2504	1.9298	23.6502	6.2047
493	M	24	0	BDL	BDL	BDL	BDL	BDL	BDL	110%*	BDL
496	F	24	2.25	4.7906	0.8523	85.9820	4.3797	0.3800	0.3750	9.5980	0.1179
497	F	4	2.25	7.1370	0.4085	37.6208	0.8387	0.5096	0.2420	5.0254	0.0972
501	M	1	2.25	6.4114	0.6837	63.2663	1.2424	0.6457	1.0096	11.1973	BDL
504	M	24	2.25	3.4445	0.2716	31.1063	0.7366	0.1520	BDL	2.3196	BDL
505	M	4	2.25	6.5663	0.7739	43.9213	8.7091	1.1300	1.3401	20.9054	0.5732
467	F	1	3.34	7.0353	1.4337	108.9275	5.7677	0.7037	0.3589	14.1832	0.1018
474	M	4	3.32	4.5530	0.6787	62.3798	0.9769	0.6810	0.1051	13.7065	2.9818
476	F	24	3.32	NS	0.7723	31.4083	0.8227	0.3541	0.0753	11.0096	0.1368
485	M	1	3.34	5.3894	1.2978	75.8182	1.5811	0.5202	0.1270	18.9590	0.1578

*Selected control samples (0 mg/m^3) were spiked with GB to determine percentage recovered.
BDL: Below detection limit (<0.05 ng/g)
NS: No sample was received.

Table 3. Results from Regenerated and Free GB Assays of PND 42 Rats (continued)

Rat No.	Sex	Sample Time (h)	Concentration (mg/m^3)	Regenerated and Free GB (ng/g)							
				Whole Blood	Heart	Lung	Liver	Kidney	Brain	Eye	Diaphragm
491	F	4	3.32	5.6716	0.6269	68.8785	2.1254	0.6023	0.1080	16.6445	0.2991
500	M	24	3.32	6.1446	1.7415	126.0829	12.0281	0.6395	0.4760	12.2233	0.6865
556	F	24	0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
557	F	1	3.34	11.6924	3.3015	172.6911	50.0753	1.3233	2.2118	26.6448	0.7088
558	F	4	3.32	7.0588	0.7038	45.3654	2.1496	1.1615	0.1441	18.5023	0.2677
559	F	24	3.32	8.1020	1.1831	109.0502	12.4974	1.5260	0.8463	14.3500	2.3423
560	F	1	3.34	8.5883	1.2533	105.0887	18.7626	1.4513	0.8139	24.9826	0.5630
561	M	4	3.32	6.2740	2.5751	61.9168	0.8113	0.7163	0.1123	14.3816	0.1823
562	M	1	3.34	5.6723	0.4152	37.8342	0.4758	0.4435	0.0735	16.5152	0.1862
563	M	24	0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
564	M	4	3.32	7.7468	0.6650	37.3145	0.9037	0.5731	0.1711	14.4358	0.3431
565	M	1	3.34	7.8334	0.4337	53.3045	0.7126	0.4287	0.1538	18.3605	0.0612
566	F	4	3.32	9.7324	2.2688	81.2916	5.8018	2.0787	1.7691	22.2586	9.4123
567	F	24	3.32	6.7096	1.6611	94.2823	8.9308	1.1453	0.6913	18.8913	2.3244
568	F	24	0	BDL	BDL	BDL	113%*	BDL	BDL	BDL	BDL
569	F	24	3.32	5.3270	4.1277	106.6401	15.2385	1.0785	0.5618	14.3709	0.3139
570	F	24	3.32	4.5734	0.4677	42.6116	1.1982	0.6034	0.0866	12.7972	0.0521
571	M	1	3.34	8.6931	1.0101	67.0300	2.9578	0.8985	0.2121	16.0548	0.3092
572	M	24	3.34	4.6203	0.4072	31.5732	0.6046	0.6023	0.1077	14.7002	0.2792
573	M	24	3.34	NS	1.0906	30.1032	0.8606	0.4962	0.0652	9.4561	0.4677
574	M	4	3.32	6.2076	0.5162	43.7994	0.9076	0.7014	0.1119	17.6946	0.3745
575	M	24	3.34	2.9874	0.2756	0.4236	26.4206	0.3081	BDL	NS	0.1630
576	F	1	3.34	8.0567	1.9315	193.7189	20.8696	2.2510	1.7371	25.7110	2.1312
577	F	1	3.34	8.3217	2.5352	166.1733	17.7542	1.8282	2.4476	25.1865	2.4065
578	F	4	3.32	4.1685	NS				0.0940	NS	
579	F	4	3.32	5.7831					0.4939		
580	F	24	3.34	5.8509	1.9689	124.7684	23.2542	1.7058	0.9144	17.9717	0.5838
581	M	24	3.34	3.0692	0.3489	24.9568	0.7720	0.3471	BDL	15.7459	BDL

*Selected control samples (0 mg/m^3) were spiked with GB to determine percentage recovered.
BDL: Below detection limit (<0.05 ng/g)
NS: No sample was received.

Table 3. Results from Regenerated and Free GB Assays of PND 42 Rats (continued)

Rat No.	Sex	Sample Time (h)	Concentration (mg/m^3)	Regenerated and Free GB (ng/g)							
				Whole Blood	Heart	Lung	Liver	Kidney	Brain	Eye	Diaphragm
582	M	4	3.32	7.2321	1.9171	93.4824	5.4517	1.3299	1.1221	24.7519	2.7121
583	M	1	3.34	4.1580	0.6339	43.5621	0.5058	0.5423	0.0855	26.6168	0.1253
584	M	24	3.34	2.7632	0.7048	29.3509	0.4910	0.2679	BDL	10.9662	BDL
585	M	24	0	86%*	BDL	BDL	BDL	BDL	BDL	BDL	BDL
586	F	4	3.32	6.2974	1.6116	165.4422	8.9424	1.3769	1.0693	38.9464	0.7163
587	F	1	3.34	6.3891	1.1356	82.0686	17.2119	0.9088	0.9838	28.0528	0.5793
588	M	1	3.34	3.4683	0.7132	56.2571	1.1427	0.4976	0.0743	32.6441	0.2598
589	M	4	3.32	4.9295	0.7719	68.2645	1.2603	0.6132	0.1106	28.5636	0.0974
590	F	24	4.94	8.5959	5.0263	142.7565	25.8151	4.0380	4.5124	21.1992	2.9992
592	F	1	4.83	8.8297	9.4257	NS	77.5467	7.1321	8.6961	21.2176	3.7392
593	F	1	4.83	7.5018	4.3431	NS	47.6051	3.2538	6.7196	30.4227	3.5013
594	F	24	4.83	8.5328	4.8135	201.7641	46.8426	6.2970	4.4956	21.8503	2.8778
595	M	24	4.83	7.0418	4.4824	69.9723	7.0233	3.0205	2.6940	14.2434	1.3506
596	M	24	4.83	NS	3.4385	98.4132	8.5643	3.0806	1.7193	17.4639	0.5944
597	M	1	4.83	7.7530	10.9775	NS	26.9908	3.8186	5.7603	22.1529	1.9964
598	M	1	4.83	7.3913	6.3064	NS	23.2011	3.7567	6.1959	20.4392	28.9302
599	M	24	4.83	7.1405	4.4592	116.7299	9.1643	4.3329	1.8751	23.3930	0.5025
602	F	1	4.83	11.0519	5.8023	NS	61.8314	6.1537	3.6278	**	0.1815
603	F	1	4.83	9.5891	8.3216	NS	9.9408	3.7616	4.0198	24.0126	1.3860
604	F	24	0	BDL	BDL	BDL	BDL	85%*	127%*	**	BDL
605	M	24	0	BDL	BDL	BDL	120%*	BDL	BDL	**	BDL
606	M	4	4.94	7.1776	3.9480	NS	2.3321	3.0389	2.6197	23.2114	0.9170
607	M	1	4.83	5.2898	2.7011	NS	2.2213	1.9188	1.7465	10.1281	0.6902
608	M	4	4.94	NS	7.3728	NS	11.2529	5.1918	3.7401	23.3217	1.2653
609	M	1	4.83	7.5334	11.7463	NS	6.6637	2.9933	3.5901	23.1258	0.5856
611	F	4	4.94	8.1357	6.1576	NS	9.2057	3.8934	4.4932	21.0955	2.7021
612	F	24	4.94	11.2587	5.7370	77.4434	17.4970	10.3101	3.0527	28.7891	4.3218

*Selected control samples (0 mg/m^3) were spiked with GB to determine percentage recovered.

**These samples were lost during assay preparation.

BDL: Below detection limit (<0.05 ng/g)

NS: No sample was received.

Table 3. Results from Regenerated and Free GB Assays of PND 42 Rats (continued)

Rat No.	Sex	Sample Time (h)	Concentration (mg/m^3)	Regenerated and Free GB (ng/g)							
				Whole Blood	Heart	Lung	Liver	Kidney	Brain	Eye	Diaphragm
613	F	24	4.94	8.3054	4.5674	105.3901	9.8348	4.6050	2.4272	17.9905	2.2108
614	F	4	4.94	9.3489	6.9861	NS	12.3481	4.1348	2.9735	23.4932	1.4204
615	M	24	4.94	6.7252	2.8212	169.3489	4.2511	2.8779	1.4793	8.3101	0.8548
617	M	1	4.83	6.5679	2.7166	NS	4.8268	2.1705	2.0295	20.5711	0.3903
618	M	24	4.94	6.0658	2.1850	77.9095	6.7666	2.3702	1.5718	17.5181	0.8651
619	M	24	4.94	4.9778	1.4374	71.1554	2.8545	1.3970	0.6857	13.3203	0.3655
620	F	4	4.94	4.4255	7.0557	NS	11.0386	3.3856	3.9633	30.4174	3.2746
621	F	1	4.83	7.1842	7.6177	NS	7.1605	6.4640	5.4405	24.8375	3.2929
629	M	1	6.61	21.2062	3.9430	228.1735	14.7299	2.7095	5.0848	15.5598	1.1859
632	M	1	6.61	49.8976	31.9572	331.9180	9.0291	8.2309	10.7514	26.5072	5.6944
639	M	4	6.56	36.9136	1.9050	177.0564	8.5334	3.9412	3.7119	26.7217	0.5845
640	M	4	6.56	22.2591	5.5766	134.7770	7.7368	NS	4.4910	20.8845	13.7025
641	M	4	6.56	28.7037	6.5094	NS	4.1650	3.6159	4.7483	18.0844	2.3991
642	M	4	6.56	34.3488	8.7887	321.5985	2.9181	6.2973	7.9049	25.6246	2.6942
643	M	4	6.56	34.7302	4.4769	148.8294	12.9604	3.9485	5.9419	NS	0.6239
644	F	4	6.56	30.7059	11.2811	246.8997	39.0395	8.7828	8.8139	23.3269	2.9477
648	F	24	0	BDL	122%*	4.3237	BDL	BDL	BDL	BDL	BDL
649	M	1	6.61	65.9162	7.2573	160.2248	10.7862	5.9094	NS	24.5391	0.9137
650	M	24	6.61	14.4245	4.9915	226.2828	3.7251	4.5433	3.6154	19.6495	0.9116
659	M	24	0	BDL	0.1356	120%*	BDL	BDL	BDL	BDL	BDL
663	M	4	6.56	44.9037	5.9154	269.8755	9.9057	6.9065	5.7907	22.7100	1.0618
672	M	24	6.56	15.8063	7.5573	161.5769	11.0754	6.3227	4.1516	29.0325	2.2549
676	F	24	6.56	17.4363	6.6052	197.8823	5.7633	5.0055	2.5233	16.8357	2.2633
677	F	24	0	BDL	0.2967	6.7058	BDL	BDL	BDL	BDL	BDL
680	M	24	0	BDL	0.0719	2.4811	BDL	BDL	BDL	BDL	BDL
681	M	24	6.56	15.9850	9.5919	132.4148	9.7331	4.5620	2.6323	23.7433	2.1705
683	M	24	6.61	17.4412	7.3503	184.5233	9.1931	5.1442	3.0655	18.0809	1.4316

*Selected control samples (0 mg/m^3) were spiked with GB to determine percentage recovered.
BDL: Below detection limit (<0.05 ng/g)
NS: No sample was received.

Table 4. Results from Regenerated and Free GB Assays of PND 70 Rats

Rat No.	Sex	Sample Time (h)	Concentration (mg/m^3)	Regenerated and Free GB (ng/g)							
				Whole Blood	Heart	Lung	Liver	Kidney	Brain	Eye	Diaphragm
294	F	1	5.37	10.1896	13.8003	166.4178	10.5459	28.1108	9.6868	47.3811	2.0374
295	F	1	5.37	8.1349	4.3370	219.6183	25.7320	10.9081	3.5280	47.5029	1.1834
296	F	4	5.37	11.4351	10.2113	189.2171	26.7417	20.9604	9.3314	30.3172	4.6908
297	F	1	5.37	8.5763	5.8323	311.7013	25.0778	14.7000	5.9086	44.3391	2.6759
298	F	4	5.37	8.1149	5.1124	283.1156	9.7455	11.8273	4.9405	52.1720	1.5882
299	F	4	5.37	11.1306	6.7422	265.1238	27.1930	18.1324	6.5596	38.9347	2.1348
684	F	4	2.85	10.5705	7.6587	308.8672	13.9717	3.6661	2.7035	24.6314	3.0846
685	F	4	2.85	14.5657	4.5029	89.1195	20.1696	3.1390	1.4591	20.5418	1.5899
686	F	4	2.85	6.8942	3.4372	204.9153	2.4881	2.2393	1.2304	18.6625	0.8669
687	F	4	2.85	7.0526	3.3184	132.6021	2.6465	1.7477	0.9808	20.6164	0.8786
688	F	4	2.85	11.3672	6.6478	263.3733	5.3481	6.0540	2.8803	34.4920	3.9664
689	F	4	2.85	3.8154	0.7019	25.6503	0.5371	0.4264	BDL	17.9932	0.4325
690	F	1	2.85	14.0157	1.0708	134.1235	1.1421	0.5562	0.2964	22.1158	0.5608
691	F	1	2.85	11.1168	0.9099	97.5398	1.0401	0.6695	0.1982	10.4669	1.0436
692	F	1	2.85	17.4294	5.0150	230.0583	7.4764	2.6075	1.4020	23.0971	4.3517
693	F	1	2.85	9.8712	2.5648	122.9945	6.0134	1.8512	0.9577	23.7481	0.7005
694	F	1	2.85	11.5465	1.7775	126.8154	9.7642	1.5071	0.9542	35.1560	0.5604
695	F	1	2.85	12.5407	5.0768	209.8384	4.4117	1.8418	2.1027	19.6250	1.1178
696	F	24	2.85	8.2256	4.5597	129.2711	24.9533	1.6978	0.6724	23.1563	0.8952
697	F	24	2.85	6.2941	2.2936	93.9017	4.0923	0.9752	0.2637	15.6691	0.2981
698	F	24	2.85	8.0553	1.0602	88.9837	2.6657	0.8253	0.3343	14.5487	0.2600
699	F	24	2.85	9.3888	4.6551	158.4006	14.0287	2.0785	0.8626	17.3809	2.0458
700	F	24	2.85	9.5271	4.8570	124.4416	6.0806	1.7565	0.6919	22.4503	0.7752
701	F	24	2.85	9.1242	5.2136	151.4494	4.4638	3.2715	1.1624	12.3319	1.0250
702	F	24	0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
703	F	24	0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
724	M	4	2.85	5.8404	0.5227	47.1660	**	0.4246	0.0872	13.6997	0.5024
725	M	4	2.85	10.1961	0.5595	45.3796	0.4462	0.4729	0.1047	18.5351	0.5754

**This sample was lost during assay preparation.
BDL: Below detection limit (<0.05 ng/g)
NS: No sample was received.

Table 4. Results from Regenerated and Free GB Assays of PND 70 Rats (continued)

Rat No.	Sex	Sample Time (h)	Concentration (mg/m^3)	Regenerated and Free GB (ng/g)							
				Whole Blood	Heart	Lung	Liver	Kidney	Brain	Eye	Diaphragm
726	M	4	2.85	9.1774	0.4582	26.4772	0.5658	0.3920	0.0714	14.6173	0.2784
727	M	4	2.85	4.5678	0.2596	27.2680	0.5748	0.3950	0.0618	12.1228	0.2830
728	M	4	2.85	6.8016	0.6586	31.6418	0.4138	0.3884	0.0599	12.1984	0.3399
729	M	4	2.85	9.1766	1.0185	30.8128	0.5464	0.4523	0.0841	9.5991	0.3168
730	M	1	2.85	17.9130	0.4838	24.8600	0.7491	0.8940	0.1142	9.5273	0.4601
731	M	1	2.85	5.3782	0.4779	35.8437	0.2613	0.4257	0.0957	12.3677	0.1481
732	M	1	2.85	14.5661	0.8269	68.9011	0.7822	0.5772	0.1560	13.2675	0.7795
733	M	1	2.85	20.8509	2.9695	94.0642	0.7755	1.0464	0.9070	28.0677	0.8826
734	M	1	2.85	17.6179	1.0003	37.6560	0.4654	0.4155	0.1051	10.9138	0.2160
735	M	1	2.85	12.4422	1.5301	68.8216	1.1588	0.8459	0.2798	13.5633	0.8311
736	M	24	2.85	5.3051	0.3639	29.5688	0.4296	0.2775	0.0778	7.8427	0.2487
737	M	24	2.85	5.3971	2.0719	19.1288	0.4669	0.3691	0.0588	10.2780	0.1421
738	M	24	2.85	3.8608	0.3115	26.9650	0.4351	0.2762	0.0853	7.9132	0.0786
740	M	24	2.85	9.0633	0.9264	65.7080	0.4976	0.7554	0.1965	11.9393	0.3693
741	M	24	0	139%*	BDL	BDL	BDL	BDL	BDL	BDL	BDL
742	M	24	0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
743	M	24	2.85	8.0504	0.4912	69.1360	0.8040	0.2308	0.0840	11.1937	0.2172
705	F	4	4.34	17.0689	6.7024	243.5101	7.3533	4.0393	5.1631	23.1257	2.0701
706	F	4	4.34	23.5192	8.7820	353.4567	22.6516	8.4567	4.9214	18.8239	5.4503
707	F	4	4.34	14.6544	8.1109	400.0278	7.6905	5.8881	3.8309	20.8133	3.9076
709	F	4	4.34	18.2944	8.0432	364.8486	5.7976	3.9843	4.4756	17.8929	2.8783
710	F	1	4.3	19.8606	8.2419	364.7488	9.1694	2.3251	NS	17.4115	5.7785
711	F	1	4.3	13.7117	2.5186	181.4805	5.9309	1.6499	0.8009	16.8075	0.8618
712	F	1	4.3	15.8062	7.5471	291.0298	19.7023	NS	5.0988	19.2900	1.4800
713	F	1	4.3	20.4745	7.0094	262.0288	9.3835	3.7157	3.3312	23.3263	1.6767
714	F	1	4.3	24.7707	10.9599	497.8647	14.8448	NS	5.8916	16.5983	4.6716

*Selected control samples (0 mg/m^3) were spiked with GB to determine percentage recovered.
BDL: Below detection limit (<0.05 ng/g)
NS: No sample was received.

Table 4. Results from Regenerated and Free GB Assays of PND 70 Rats (continued)

Rat No.	Sex	Sample Time (h)	Concentration (mg/m^3)	Regenerated and Free GB (ng/g)							
				Whole Blood	Heart	Lung	Liver	Kidney	Brain	Eye	Diaphragm
715	F	1	4.3	19.1338	6.8449	651.7517	5.4510	NS	3.7019	20.8015	1.6051
717	F	24	4.34	13.4290	8.7264	201.1080	10.0362	3.7410	1.9796	16.5466	2.1210
718	F	24	4.34	13.8573	7.7718	188.4956	9.8351	5.7236	2.4636	13.0171	3.1915
719	F	24	4.3	13.4562	11.2258	214.1708	10.1608	4.2370	2.1360	19.8885	1.8938
720	F	24	4.3	14.6106	6.6504	188.2079	7.6102	4.5668	1.8408	16.4990	1.7190
721	F	24	4.3	17.4256	6.4639	309.9033	2.5705	3.9103	2.2917	15.4374	2.1929
722	F	24	0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
723	F	24	0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
744	M	4	4.34	19.9764	3.7577	156.7967	2.1751	1.8196	1.8351	14.8138	0.6020
745	M	4	4.34	16.4326	1.4742	87.2626	1.7229	0.7049	0.3336	9.2890	0.4141
746	M	4	4.34	13.8419	1.0700	47.0507	0.6591	0.4241	1.0826	21.6143	0.1870
747	M	4	4.34	15.8964	3.0195	119.2383	2.2211	1.1500	0.1530	30.7398	0.6188
748	M	4	4.34	10.7609	5.2597	178.1244	1.7105	1.7963	1.5862	18.8602	1.2376
749	M	4	4.34	13.0319	2.4153	74.3978	2.8790	1.1140	0.7965	26.7519	0.4139
750	M	1	4.3	12.9617	2.2825	101.6354	1.8981	0.7121	0.7717	20.9287	0.2502
751	M	1	4.3	14.2291	2.0221	70.4555	1.4962	NS	0.2785	18.6376	0.2736
752	M	1	4.3	19.8352	4.8905	149.7019	7.8500	NS	1.8376	18.5901	0.6228
753	M	1	4.3	5.9770	0.4856	25.3447	0.6732	NS	0.0771	24.1641	0.2070
754	M	1	4.3	9.8251	0.4090	33.0835	0.3319	NS	0.0766	9.0437	0.1979
755	M	1	4.3	24.6101	1.7354	115.7397	2.6957	0.7351	0.7122	17.7742	1.8963
756	M	24	4.34	7.0756	1.6216	87.5078	1.7777	0.8995	0.3570	14.0666	0.4800
757	M	24	4.34	8.9209	4.0206	128.6088	6.4224	1.7705	1.2008	12.5225	1.1627
758	M	24	4.34	12.3066	6.2647	150.6566	5.7830	1.9258	1.3456	13.2484	0.9278
759	M	24	4.3	7.1656	0.8038	67.6564	0.7431	0.4887	0.1488	14.1011	0.1792
760	M	24	4.3	10.0050	1.1671	71.7491	2.4286	0.6607	0.1947	14.2382	0.2385
761	M	24	4.3	7.9290	5.5444	106.2105	1.4917	1.4237	0.9592	16.0538	0.6775
762	M	24	0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL
763	M	24	0	BDL	BDL	BDL	BDL	BDL	BDL	BDL	BDL

BDL: Below detection limit (<0.05 ng/g)
NS: No sample was received.

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ACRONYMS AND ABBREVIATIONS

ACh	acetylcholine
AChE	acetylcholinesterase enzyme
ANOVA	analysis of variance
BARDA	Biomedical Advanced Research & Development Authority
BDL	below detection limit
CAS	Chemical Abstract Service
CASARM	chemical agent standard analytical reagent material
CE	collision energy
CI	chemical ionization
CPF	chlorpyrifos
CWNA	chemical warfare nerve agent
ECBC	Edgewood Chemical Biological Center
GA	ethyl *N*,*N*-dimethylphosphoramidocyanidate; tabun
GB	isopropyl methylphosphonofluoridate; sarin
GC	gas chromatography
GC-MS/MS	gas chromatography–mass spectrometry/mass spectrometry
GD	pinacolyl methylphosphonofluoridate; soman
GF	cyclohexyl methylphosphonofluoridate; cyclosarin
IACUC	Institutional Animal Care and Use Committee
IPA	2-propanol
IS	internal standard
LCt_{50}	lethal concentration with 50% chance of causing death
MRM	multiple reaction monitoring
m/z	mass-to-charge ratio
NMR	nuclear magnetic resonance
NS	no sample received
OP	organophosphorus (pesticides)
PBPK/PD	physiologically based pharmacokinetic/pharacodynamic
PND	postnatal day
R^2	coefficient of determination
r-GB	regenerated GB
Russian VX	*O*-isobutyl *S*-[(2-diethylamino)ethyl] methylphosphonothioate
SPE	solid-phase extraction
VX	*O*-ethyl *S*-(2-diisopropylaminoethyl) methylphosphonothioate

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